Amendments to the Drawings:

The attached replacement sheet of drawings includes changes to Fig. 1 and replaces the original sheet including Fig. 1.

In Figure 1, Applicants respectfully request amendment of the cobalt atom from Co^{+} to Co^{+3}.

Attachments following last page of this Amendment:

Replacement Sheet (1 page)
Annotated Sheet Showing Change(s) (1 page)

REMARKS

Applicant hereby requests amendment of Figure 1 in the above patent in accordance with the attached request.

In Figure 1, Applicants herein amend the structure to replace the cobalt center marked as "Co^{+}" to "Co^{+3}." Applicants believe that the error in the original drawing was the result of an inadvertent clerical error when the figure was adapted from *The Merck Index*, Merck and Co. (11^{th} Ed., 1989), as detailed in the specification at paragraph 2.

Applicants believe that the error and the appended correction would have been apparent to one of ordinary skill in the art from the specification and prosecution history in view of *The Merck Index*, a copy of which is attached hereto. As one of ordinary skill in the art would recognize, the overall charge on the cobalt atom, currently depicted in the drawing as "Co^{+}," can change with the X substituent (and indeed is Co^{+} when X is CN, as in *The Merck Index*).

Similarly, one of ordinary skill in the art would recognize that the oxidation state of the cobalt center is Co^{+3}. Applicants respectfully assert that one of ordinary skill in the art would understand that the charge, but not the oxidation state, would vary according to the identity of X, as disclosed in the present application. The oxidation state, as proposed in the Replacement Sheet to be depicted as "Co^{+3}," however, will be independent of the identity of X. Applicants therefore believe one of ordinary skill in the art would recognize the proposed amended structure to correctly represent the claimed structure, rather than the Figure 1 present at the time of filing. Such a change is further supported by the attached references: (1) *B_{12} **vol. 1***, D. Dolphin, Ed. (1982, pp. 17-21), a reference cited by the above-referenced *The Merck Index*, which contains a structure of adenosylcobalamin with the Co^{+3} center clearly illustrated; and (2) Bernhauer, K., Müller, O., and Wagner, F., *Angew. Chem. Int. Ed.* **1964**, 3(3), 200 (*see, e.g.*, page 205, section III, which states: "[x]-ray diffraction studies on the cobalamin coenzyme have shown that, like vitamin B_{12}, it [adenosylcobalamin] contains Co^{3+}."). Applicants respectfully request introduction of the amended Figure 1. No new matter has been added.

No fee is believed due. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 9/27/08

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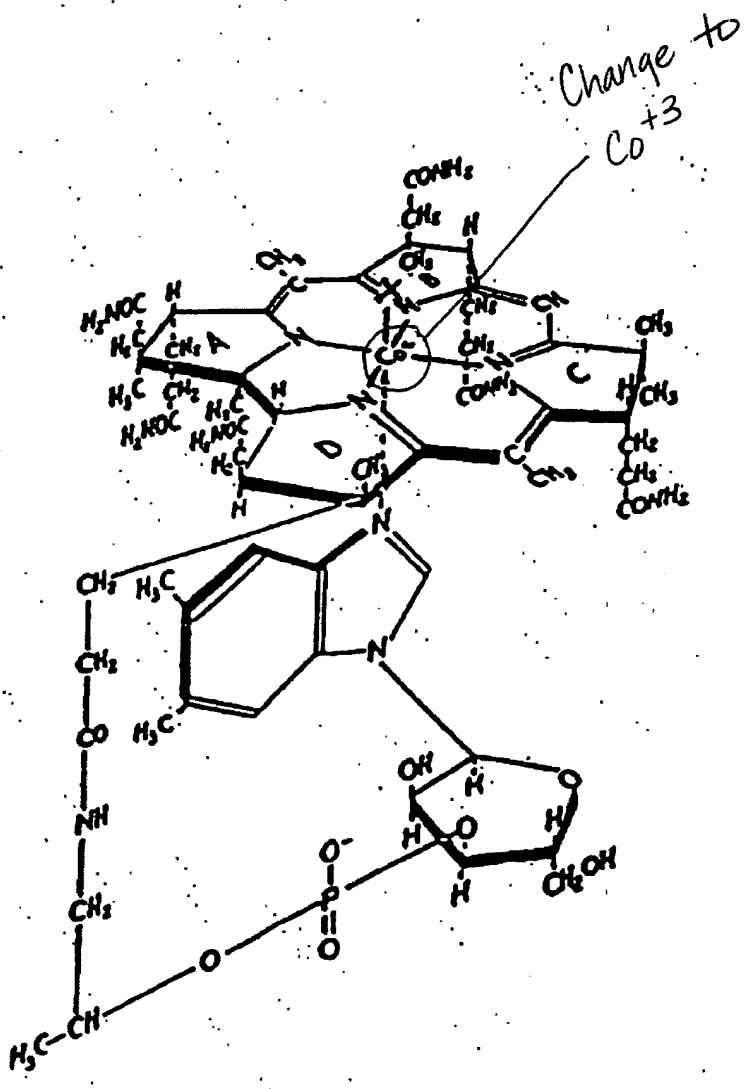
Annotated Sheet Showing Change(s)

Figure 1



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AN ENCYCLOPEDIA OF
CHEMICALS, DRUGS, AND BIOLOGICALS

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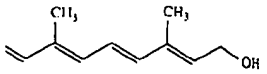
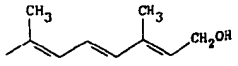
mol wt 286.44. C 83.86%, H 10.56%, O
e animal organism (not in plants); caro-
d into vitamin A by the liver. Extracted
where it occurs mostly in esterified form;
t al., Helv. Chim. Acta 14, 1036, 1431
16, 625 (1933); Heilbron et al., Biochem
32). Stereochemistry: see L. Zechmeis-
267 (1944). Identity of lard-factor and
Harris, Science 120, 391 (1954). Total
A from β-ionone and a propargyl ha-
it, U.S. pat. 3,060,229 (1962 to Bayer).
nal: Wendler et al., J. Am. Chem. Soc.
n, Kapp, U.S. pat. 2,972,634 (1961 to
ama, A. Ishida, Chem. Letters 1975,
synthesis of all (E)-form: P. S. Man-
him. Acta 59, 567 (1976); A. Fischli et
dillo et al., J. Chem. Soc., Perkin Trans.
ology: J. J. Kamm, J. Am. Acad. Der-
). Comprehensive monograph of the
physiology of vitamin A and its pro-
rell, R. S. Harris, The Vitamins, Vol. I,
w York, 2nd ed., 1967) 570 pp. Book
-2, M. B. Sporn et al., Eds. (Academic
84). See also: Neovitamin A.



propylene oxide or petr ether. Solvat-
e polar solvents, such as methanol or
-64°. Distills at 120-125° at 5 × 10⁻³
410 (calculated from refractive indexes
neral oil). uv max: 324-325 nm ($E^{1\%}_{1cm}$
on, J. Am. Chem. Soc. 64, 2407 (1942)).
vater or glycerol; sol in abs alcohol,
, ether, fats and oils. Ultraviolet light
and its solns which exhibit a charac-
ence. The free alcohol is sensitive to
solns of it are quite stable. Esters of
table to oxidation. LD_{50} (10 day) in
-p.; 2570 orally (Kamm).
pale yellow prismatic crystals from
uv max (ethanol): 326 nm ($E^{1\%}_{1cm}$
04 × 10⁶ I.U./g. LD_{50} (10 day) oral-
kg (Kamm).
, *Arovit, Optovit-A,* is the ester pre-
oils. Amorphous or cryst. mp 28-
325-328 nm ($E^{1\%}_{1cm}$ 975). Biopotency
LD_{50} (10 day) in mice, rats (mg/kg):
nrm).
it of vitamin A is equal to 0.30 micro-
ivalent to 0.344 microgram of vitamin
biopotency 3.33 × 10⁶ I.U./g.
ophthalmic vitamin.
utritional factor.

3,4-Didehydroretinol; retinol$_2$; deby-
ol wt 284.42. C 84.45%, H 9.92%, O
h opsin to form the visual pigment
esh-water fish. A mixture of stereo-
ce liver oils: Shantz, *Science* 108, 417
z, Brinkman, *J. Biol. Chem.* 183, 467
Chem. Soc. 1952, 503. Synthesis and
isomers: Schwieter, *Chimia* 14, 362
cta 45, 517, 528, 541, 548 (1962);
vol. I, W. H. Sebrell, R. S. Harris,
New York, 2nd ed., 1967) *passim.*



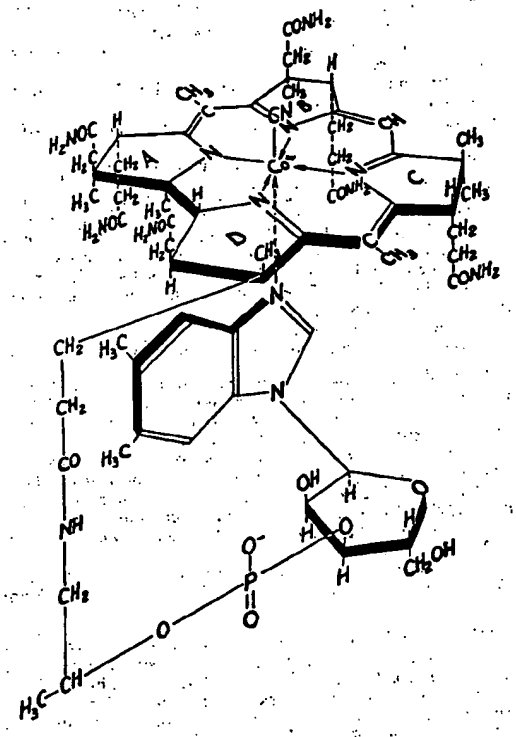
stereoisomeric mixture, golden yellow oil. Readily affected by oxygen. uv max (ethanol): 351, 287 nm ($E^{1\%}_{1cm}$ 1460, 1000). Has about 40% of the bioactivity of cryst vitamin A_1. Stereoisomers: All-*trans*, mp 17-19°; 13-*cis*, mp 73-74°; 9-*cis*, mp 77-79°; 9,13-di-*cis*, mp < −30°.

Vitamin B_{10} and Vitamin B_{11}. Two factors reported to be necessary for growth and feathering of chicks: Briggs *et al., J. Biol. Chem.* 148, 163 (1943). Prepn from culture filtrates of *Mycobacterium tuberculosis*: Mills *et al., Proc. Soc. Exp. Biol. Med.* 56, 240 (1944).

Vitamin B_{12}. Cyanocobalamin; 5,6-dimethylbenzimidazolyl cyanocobamide; cobinamide cyanide phosphate 3'-ester with 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole inner salt; LLD factor; Lactobacillus lactis Dorner factor; extrinsic factor; antipernicious anemia principle; Anacobin; Antipernicin; Bedoce; Bedodeka; Bedoz; Behepan; Benabic; Berubigen; Betalin-12; Betolvex; Bevatine-12; Bevidox; Bexibi; Bexil; Biocobalamine; Biocres; Bitevan; B-Telve; B-Twelve; Byladoce; Claretin-12; Cobalin; Cobamin; Cobamline; Cobione; Covit; Crystamin; Cycobemin; Cycolamin; Cytakvonimel; Cytacon; Cytamen; Cytobion; Distivit (B_{12} peptide); Dobetin; Docemine; Docibin; Docigram; Docivit; Dodecabec; Dodecavite; Dodex; Ducobee; Duodecibin; Emlok; Emociclina; Eritrone; Erycytol; Erythrotin; Euhaemon; Fremin; Hemo-B-Doze; Hemomin; Hepagon; Hepavis; Hepcovite; Hydoxamin; Hydroxobase; Macrabin; Megabion (Indian); Megalovel; Milbedoce; Millevit; Nagravon; Normocytin; Peraemon; Pernaevit; Pernipur; Plecyamin; Poyamin; Redamina; Redisol; Rhodacryst; Rubesol; Rubivitan; Rubramin; Rubripca; Rubrocitol; Sytobex; Vibalt; Vibisone; Virebra; Vitarubin; Vita-Rubra; Vitral. $C_{63}H_{88}CoN_{14}O_{14}P$; mol wt 1355.38. C 55.83%, H 6.54%, Co 4.35%, N 14.47%, O 16.53%, P 2.28%. A cobalt containing coordination compd produced by intestinal microorganisms. Found also in soil and water, the richest sources being activated sewage sludge (see Milorganite), manure, and dried estuarine mud. Higher plants do not concentrate vitamin B_{12} from the soil and so are a poor source as compared with animal tissues. First isolated from liver: Rickes *et al., Science* 107, 396 (1948). Also from cultures of *Streptomyces griseus:* Rickes *et al., ibid.* 108, 634 (1948); Rickes, Wood, U.S. pats. 2,563,794 and 2,703,302-3 (1951 and 1955 to Merck & Co.); Smith, Hull, U.S. pat. 2,951,017 (1960 to Distillers Co.); McDaniel, Long, U.S. pats. 3,000,793 and 3,018,225 (1961, both to Merck & Co.). From sewage sludge: Van Melle, U.S. pat. 3,057,851 (1962 to Armour); Bernhauer *et al.*, U.S. pat. 3,120,509 (1964 to Hoffmann-La Roche). Absorption through the intestinal wall is dependent on the presence of Castle's Intrinsic Factor, *q.v.* Although requirement for the vitamin is minute, deficiency states have been observed in individuals who abstain from all animal products.

Nomenclature: IUPAC rules, *Pure Appl. Chem.* 48, 497 (1976). *Cobalamin* refers to all of the molecule except the cyano group. The fundamental ring system without cobalt or side chains is called *corrin* and the octadehydrocorrin is called *corrole.* The Co-contg heptacarboxylic acid resulting from hydrolysis of all the amide groups without the CN and the nucleotide, is designated *cobyrinic acid.* The corresp hexacarboxylic acid which still retains the 2-hydroxypropionamide group on a side chain is called *cobinic acid* and the hexacarboxylic acid having the ribofuranosidophosphoryl-propylamide side chain is called *cobamic acid.* Thus *cobamide* is the hexaamide of cobamic acid, *cobyric acid* is the hexamide of cobyrinic acid and *cobinamide* is the hexamide of cobinic acid.

Structure announced by A. Todd and team (Cambridge), D. Hodgkin and team (Oxford), and E. L. Smith (Glaxo), see *Nature* 176, 325, 328, 551 (1955); 178, 64 (1956). X-ray structure analysis: D. C. Hodgkin, *Fortschr. Chem. Org. Naturst.* 15, 167-227 (1958). Stereochemistry: Stora, *Bull. Soc. Chim. France* 1959, 1421. Total synthesis: Woodward, *Pure Appl. Chem.* 33, 145 (1973). Monograph: Smith, *Vitamin B_{12}* (Methuen & Co., London, 3rd ed., 1965); B. Zagalak, W. Friedrich, Eds. *Vitamin B_{12}* (de Gruyter, New York, 1979). *Review:* several authors in *The Vitamins,* vol. 2, W. H. Sebrell, R. S. Harris, Eds. (Academic Press, New York, 2nd ed., 1968) pp 119-259; B. T. Golding in *Comprehensive Organic Chemistry* vol. 5, E. Haslam, Ed. (Pergamon, New York, 1979) pp 549-584. Comprehensive description: J. Kirschbaum in *Analytical Profiles of Drug Substances* vol. 10, K. Florey, Ed. (Academic Press, New York, 1981) pp 183-288. Book: *B_{12}* vols. 1 and 2, D. Dolphin, Ed. (Wiley-Interscience, New York, 1982) 672 and 506 pp.



Note: Structure reproduced through the courtesy of John Wiley & Sons, Inc. from E. Lester Smith, *Vitamin B_{12}* (3rd ed., 1965) p. 32.

Hygroscopic, dark red crystals. When exposed to air, may absorb about 12% water. The hydrated crystals are stable to air. Darkens at 210-220°. Not melted at 300°. $[\alpha]^{23}_{546}$ −59 ± 9° (dil aq soln). Absorption max (water): 278, 361, 550 nm ($A^{1\%}_{1cm}$ 115, 204, 64). Odorless and tasteless. One gram dissolves in about 80 ml water. Aq solns are neutral, maximum stability in the pH range 4.5-5. Solns in this pH range can be autoclaved for 20 min at 120°. Soluble in alc. Insol in acetone, $CHCl_3$, ether. Aq solns decomp in the presence of acacia, aldehydes, ascorbic acid, ferrous gluconate, ferrous sulfate, vanillin; are stabilized by the addn of ammonium sulfate. Talc has a tenacious affinity for vitamin B_{12}; although this is not an incompatibility, it precludes the use of talc as a filter aid or lubricant for tablets, particularly in view of possible assay difficulties.

Co-methylcobalamin, $C_{63}H_{91}CoN_{13}O_{14}P$, *mecobalamin, cobinamide Co-methyl deriv. hydroxide dihydrogen phosphate (ester) inner salt 3'-ester with 5,6-dimethyl-1-α-D-ribofuranosyl-1H-benzimidazole, cobaltmethyl-5,6-dimethylbenzimidazolecobalamin, methyl vitamin B_{12}, Algobaz, Hitocobamin-M, Lyomethyl, Methycobal, Methylcobaz.* Prepn: O. Müller, G. Müller, *Biochem. Z.* 336, 299 (1962); D. Dolphin, *Methods Enzymol.* 18, Pt. C, 34 (1971); M. Tohda *et al., et al.,* Ger. pat. 2,019,176 (1971 to Eisai), *C.A.* 76, 46473a (1972); D. Autissier *et al., Bull. Soc. Chim. France* 1980, part 2, 192. Bright red crystals from water/acetone. uv max (pH 7): 522, 342, 266 nm (ε 9357, 14416, 19897); (0.1*N* HCl): 462, 304, 264 nm (ε 9599, 22855, 24737).

THERAP CAT: Hematopoietic vitamin.

THERAP CAT (VET): Nutritional factor (growth and antianemic factor).

B_{12}

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Chapter Two

Nomenclature

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The first international rules or recommendations concerning the naming of corrinoids related to vitamin B_{12} appeared in 1951 in the proceedings of the 16th Conference of IUPAC (1). Under the heading "Nomenclature of the Vitamins," and referring to a 1949 report by B. C. P. Jansen (2), the Commision on the Nomenclature of Biological Chemistry (CNBC) of IUPAC stated that "the following rules are adopted. (*a*) The group of vitamins possessed of B_{12} activity shall be designated collectively as cobalamin. (*b*) The pure substance hitherto known as vitamin B_{12} shall be designated cyano-cobalamin. (*c*) The pure substance hitherto known as vitamin B_{12b} shall be designated hydroxo-cobalamin. (*d*) The pure substance hitherto known as vitamin B_{12c} shall be designated nitroso-cobalamin." This simple statement was repeated (in French) in the proceedings of the 18th (1955) Conference (3) with a change of nitroso to nitrito.

In the meantime, the IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) had been considering the corrinoids, and in the "Blue Book" of 1959 (4), which was concerned almost exclusively with hydrocarbons and fundamental heterocyclic systems, there are tentative rules for "the vitamin B_{12} field." The numbering chosen for the corrin nucleus and its derivatives is that appearing in *The Ring Index* (1940) (5), which does not correspond to the current system (position 1 was where the present 19 is found, and 20 was not omitted, so that the nitrogens were numbered 20-23, rather than, as now, 21-24). Cobyrinic, cobinic, and cobamic acids, and cobinamide and cobamide, were defined and formulated as at present. Vitamin B_{12}, "factor A," and vitamin $B_{12(III)}$ were defined as substituted (-yl) cobamide cyanides, and the replacement of cobalt by other metals was considered. No "cobalamin" terms appear in this document, but the full term for vitamin 12b uses "aquo" rather than "hydroxo."

The report of CNBC to the 20th Conference (6) notes that a report by a committee under the chairmanship of Professor Kühnau (Hamburg) on this subject was

received by the Coordinating Committee of IUPAC and IUB and transmitted to CNBC after having "received further extensive study by CNOC" [clearly a reference to (4)]. The result of this effort was approved by the Council of IUPAC in 1959 and published in the open literature in 1960 as part of "Definitive Rules for the Nomenclature of Amino Acids, Steroids, Vitamins, and Carotenoids" (7). In this report, corrin is renumbered to correspond with porphyrin (the bond between C–19 and C–1 subtends the missing C–20, and the nitrogens carry locants 21-24). The only other difference from CNOC's 1957 document (4) is in the inclusion of cyanocobalamin, aquocobalamin, and nitrito-cobalamin (for vitamins B_{12}, B_{12b}, and B_{12c}, respectively) from the earlier CNBC document. (B_{12a} does not appear until later.)

In 1964, when the Coordinating Committee of IUPAC and IUB became the IUPAC-IUB Commission on Biochemical Nomenclature (CBN), corrinoid nomenclature was reviewed, corrected, somewhat expanded, and then published in at least seven journals and in three languages (8) in 1965-1966. The more significant changes were to name vitamins B_{12a} and B_{12b} as (the tautomeric compounds) aquocobalamin and hydroxocobalamin, 12r and 12s as cob(II)alamin and cob(I)alamin (the error in including "cyano" before the last two was corrected in the 1973 revision), and to give a systematic name for "pseudovitamin B_{12}": α-adenyl-*Co*-5′-deoxyadenosylcobamide. With the exception of those corrinoids in which metals other than cobalt are in the central position (ferrobamic, etc.), and the equivalency of cobyric acid and

Table 1

	Specific Names, in Increasing Complexity	
1. Skeleton (porphyrin nucleus minus C-20)	Corrin	
	Heptaacid	Heptaacid hexaamide
2. 1, with standard side chains and with cobalt	Cobyrinic acid	Cobyric acid
3. 2, with D-1-amino-2-propanol at position f	Cobinic acid	Cobinamide
4. 3, with D-ribofuranose 3-phosphate at position 2 of the aminopropanol	Cobamic acid	Cobamide
5. 4, with heterocyclic base attached by *N*-glycosyl link at position 1 of ribose and attached as an α ligand to cobalt		Aglyconylcobamide
6. Many B_{12} vitamins and derivatives, in which heterocyclic base is 5,6-dimethylbenzimidazole, are given the trivial name "cobalamin"		Cobalamin
7. B_{12} coenzymes, compounds in which a further organic group (X-yl) is covalently β-ligated to cobalt (see Fig. 1)		X-ylcobalamin; (*Co*α-ligandyl)-(*Co*β-X-yl)cobamide

Factor V_{1a}) the 1965-1966 recommendations are summarized in Table 1, taken from the 1973 revision.

The latest and last revision (1973) (9) clarifies some sections of the 1965-1966 document (8) and adds a section on symbols and abbreviations. The main additions and changes are as follows:

1. The derivation of "corrin" from "core" (of B_{12}), not from cobalt, is made explicit. However, the "cob" in cobalamin, and so forth, is derived from cobalt.
2. "Octadchydrocorrin" replaces the (incorrect) "tetradehydrocorrin" and the inconvenient "tetrakis-(didehydro)corrin," and is named "corrole."
3. A formal nomenclature for compounds containing unusual ligands attached to the cobalt, or aglycons that do not bridge the ribose and the cobalt, is considered (see items 5 and 7 in Table 1).
4. Stereochemistry, including α and β liganding positions of the cobalt, is made explicit.
5. The cofactors (coenzymes) are given explicit chemical names (see 7 in Table 1), and the 5′-deoxy-5′-adenosyl radical that is a part of coenzyme B_{12} (see Fig. 1) is replaced by adenosyl (for brevity as well as to avoid confusion with 2′-deoxyadenosyl, also by analogy with *S*-adenosylmethionine).

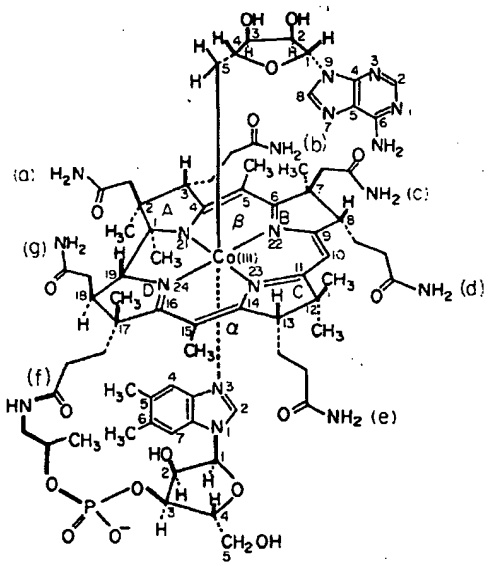

Figure 1 Coenzyme B_{12} Adenosylcobalamin. *Coα*-[α-(5,6,-Dimethylbenzimidazolyl)]-*Coβ*-adenosylcobamide. *Coα*-[α-(5,6-Dimethylbenzimidazolyl)]-*Coβ*-(5′ deoxy-5′-adenosyl)cobamide. An attempt to indicate stereochemical properties is made by using broken lines to indicate groups lying below the plane of the corrin nucleus.

6 B-12 is recommended over B_{12}; the former is desired by the International Union of Nutritional Sciences and the American Institute of Nutrition and is more amenable to computer information systems.

The appendix on abbreviations was "inspired by the burgeoning literature concerning corrinoid compounds, many of which have long and unwieldy names—a fact that has led to a variety of *ad hoc* abbreviations that, in turn, has led to difficulties for the reader. . . . In particular, the use of DBC, DMBC, etc., is discouraged, as is the use of B-12 except as vitamin B-12, coenzyme B-12, and 'factor' terms."

The appendix states that "in accordance with several preceding CBN documents (10-12), as well as with standard chemical practice, . . . abbreviations [should be] constructed by assembling symbols representing the various radicals involved, rather than from combinations of letters drawn haphazardly from the complete names of the compounds. The use of symbols reflects the actual structure of a compound [which is the function of chemical nomenclature] and facilitates the writing of equations for its chemical transformations."

Table 2

CN-Cbl	Cyanocob(III)alamin (vitamin B_{12})
AdoCbl	Adenosylcob(III)alamin
PrCbl	*n*-Propylcob(III)alamin; methyl-, etc., similarly
(Ade)(Pr-2)Cba or (Ade)Pr^{i}-Cba[a]	*Coα*-[α-(Aden-9-yl)]-*Coβ*-isopropylcobamide
(Bza)MeCba[b]	*Coα*-(α-Benzimidazolyl)-*Coβ*-methylcobamide
2-(MeOOC)EtCbl	(2-Methoxycarbonylethyl)cob(III)alamin
(Ade-7)AdoCba[a]	*Coα*-[α-(Aden-7-yl)]-*Coβ*-adenosylcobamide
(2-SHAde-7)AdoCba[a]	*Coα*-[α-(2-Thiaaden-7-yl)]-*Coβ*-adenosylcobamide
(5-MeOBza)MeCba	*Coα*-(5-Methoxybenzimidazolyl)-*Coβ*-methylcobamide[d]
(2-MeAde-7)CN-Cba[a]	*Coα*-[α-(2-Methyladen-7-yl)]-*Coβ*-cyanocobamide
(Ade)CN-Cba[a]	*Coα*-[α-(Aden-9-yl)]-*Coβ*-cyanocobamide (pseudovitamin B_{12})
(Ade)OH-Cba[a]	*Coα*-[α-(Aden-9-yl)]-*Coβ*-hydroxocobamide (hydroxopseudovitamin B_{12})
(Ade)MeCba[a]	*Coα*-[α-(Aden-9-yl)]-*Coβ*-methylcobamide
[4-(Ade-9)Bu]Cbl[c]	[4-(Aden-9-yl)butyl]cob(III)alamin
(6MeSPur)AdoCba	*Coα*-(α-6-Methylthiopurinyl)-*Coβ*-adenosylcobamide

[a] Ade alone represents adenine bonded to the ribosyl moiety through its 7 position (i.e., a 7-α-D-ribofuranosyladenine). Bonding to the cobalt is thus through N-9. When these positions are reversed, Ade-7 and aden-7-yl are used (i.e., the locant specifies the N linked to cobalt).

[b] Bza = benzimidazolyl.

[c] As this is a cobalamin, the adenine residue is not in the *Coα* position, but is attached (-9-yl) to a but-4-yl residue that is in turn linked to the β position of the cobalt. Named as a cobamide, it would be (Me_2Bza)-[4-(Ade-9)Bu]Cba.

[d] Factor III_m

The new symbols introduced are Cby, Cbi, Cba, and Cbl for cobyric acid, cobinamide, cobamide, and cobalamin (or B_{12}), respectively, and aq for aqua; the other symbols that appear in this appendix are well known (e.g., Me, Bu, Pe, Hx for simple alkyl radicals, prefixed by c for "cyclic"; Ado for 5′-deoxy-5′-adenosyl, etc.). A system for designating ligands in the α and β positions is presented (see Table 2). Also considered are abbreviations for corrinoids having radicals other than 5,6-dimethylbenzimidazolyl in the α position, for those having alterations or substitutions in the corrin residue, and for metal replacement and isotopic labeling.

ACKNOWLEDGMENTS

The systems discussed above and the examples listed in Table 2 were developed with the assistance of many leaders in the field of corrinoid research, most of whom appear as authors in this treatise, but special acknowledgment is made of the assistance of B. M. Babior to W. E. Cohn, who was, at that time, the secretary of CBN and was charged with the completion of the revision of the corrinoid document.

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4 "The Vitamin B_{12} Field," in *Nomenclature of Organic Chemistry*, issued by the IUPAC Commissions on the Nomenclature of Organic Chemistry and the Nomenclature of Biological Chemistry in July 1957, Thornton Butterworth Ltd., London, 1968, pp. 85–87.

5 *The Ring Index*, A. M. Patterson and L. T. Capell, Eds., Van Nostrand Reinhold, New York, 1940.

6 "Report of the Commission on Biochemical Nomenclature," in *Union Int. Chim. Pure Appl., C. R. 20th Conf.*, 1959.

7 *J. Am. Chem. Soc.*, 82, 5582–5583 (1960).

*8 *J. Biol. Chem.*, 241, 2992 (1966); *Biochem. J.*, 102, 15 (1967); *Arch. Biochem. Biophys.*, 118, 505 (1967); *Europ. J. Biochem.* 2, 6 (1967); *Hoppe-Seyler's Z. Physiol. Chem.*, 348, 266 (1967); *Bull. Soc. Chim. Biol.* 49, 325 (1967); *Biochim. Biophys. Acta* 117, 285 (1966).

*9 *Arch. Biochem. Biophys.* 161 (No. 2), iii (1974); *Biochem. J.* 147, 1 (1957); *Biochemistry* 13, 1555 (1974); *Europ. J. Biochem.* 45, 7 (1974); *Pure Appl. Chem.*, 48, 495 (1976).

*Reprints are available from the Office of Biochemical Nomenclature, W. E. Cohn, Director, Biology Division, Oak Ridge National Laboratory, Box Y, Oak Ridge, Tennessee.

groups are located at the correct positions and are activated by *o*-hydroxy or *o*-methoxy groups, the desired alkaloids are formed in good yield and with hardly any by-products.

The greater the complexity of the natural products which can be isolated and structurally identified by modern techniques, the more important does it become to learn to synthetize them as simply and as rapidly as in the cell. The photosynthesis experiments of *Calvin* [56] have shown that starting from CO_2, algae can perform a total synthesis of complicated natural products within ten seconds. Only by imitating such synthetic methods can the increasing demand for physiologically active biological products be met more efficiently than by the time-consuming extraction from the plant cell.

We wish to offer our sincere thanks to the Fonds der Chemischen Industrie, the Deutsche Forschungsgemeinschaft, and to the Elberfeld Works, of Farbenfabriken Bayer for their generous support of this investigation.

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German version: Angew. Chem. 75, 957 (1963)

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New Chemical and Biochemical Developments in the Vitamin B_{12} Field

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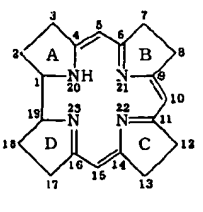
LEHRSTUHL FÜR BIOCHEMIE DER TECHNISCHEN HOCHSCHULE STUTTGART (GERMANY)

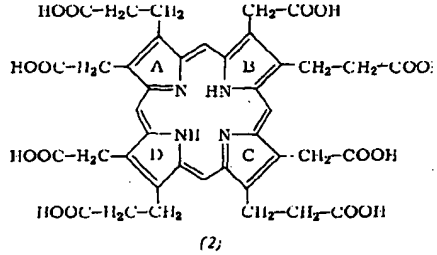
About 8000 publications have appeared in the 15 years following the isolation of crystalline vitamin B_{12} by *Folkers* and coworkers [1] in the USA and by *E. L. Smith* and *Parker* [1a] in England (see reviews [1b–8]). This field has recently received new impetus because of partial chemical syntheses and the discovery and elucidation of the structures of coenzyme forms of the Vitamin B_{12} group.

A. Nomenclature [9, 10]

Vitamin B_{12} contains a macro-ring with four nitrogen atoms. This macro-ring was named corrin *(1)*. Compounds containing this ring system are called corrinoids. All the corrinoids found so far in nature contain cobalt as the central atom. They also have acetic and



(1)



(2)

[1] *E. L. Rickes, N. G. Brink, F. R. Koniuszy, T. R. Wood,* and *K. Folkers,* Science (Washington) *107*, 396 (1948).

[1a] *E. L. Smith* and *L. F. J. Parker,* Biochem. J. *43*, Proc. VIII (1948).

[1b] *H. Knobloch:* Chemie und Technik der Vitamine. 3rd Edit., Enke, Stuttgart 1955, p. 266.

[2] *W. Stepp, J. Kühnau,* and *H. Schroeder:* Die Vitamine und ihre klinische Anwendung. Enke, Stuttgart 1957, Vol. 2, p. 557.

[3] Vitamin B_{12} und Intrinsic Factor, 1. Europ. Symposium, Hamburg 1956. Enke, Stuttgart 1957.

[4] *W. Friedrich* and *K. Bernhauer* in *K. Fr. Bauer:* Medizinische Grundlagenforschung. Thieme, Stuttgart 1959, Vol. 2, p. 662.

[5] *E. L. Smith:* Vitamin B_{12}. 2nd Edit., Methuen, London 1963.

[6] Vitamin B_{12} und Intrinsic Factor, 2. Europ. Symposium, Hamburg 1961. Enke, Stuttgart 1962.

[7] *W. Friedrich* in *R. Ammon* u. *W. Dirscherl:* Fermente, Hormone, Vitamine. Thieme, Stuttgart, Vol. 3, in the press.

[7a] Conference on Vitamin-B_{12}-Coenzymes, New York, April 1963; Ann. N.Y. Acad. Sci., in the press.

[8] Reviews on Vitamin B_{12} in annual periodicals, *e.g.* Ann. Rev. Biochem., Vitamins and Hormons, Ann. Rev. Microbiol., *etc.*

[9] IUPAC Nomenclature of Biological Chemistry, J. Amer. chem. Soc. *82*, 5582 (1960).

[10] *E. L. Smith* in [6], p. 764.

propionic acid groups in the same positions as type III porphyrins and are especially similar in this respect to uroporphyrin III *(2)*. However, ring C of the corrinoids has a methyl group instead of an acetic acid residue.

Cobyrinic acid *(3)* has six additional methyl groups –which are underlined in formula *(2a)* of the basic skeleton [*] – and six double bonds. All further compounds of this series can be named as derivatives of cobyrinic acid. Trivial names are used for the most important substances [formulae *(3)* to *(10)*]: When all the carboxyl groups except the one at position f in *(2a)* are amidated, the compound is called cobyric acid *(4)* [10] (Factor V_{1a}). In cobinic acid *(5)*, the carboxyl group at f is amidated with D_g-(–)-1-aminopropan-2-ol, while the other carboxyl groups are free. In cobinamide *(6)*, the f-carboxyl group is amidated with 1-aminopropan-2-ol and all the others are amidated with ammonia. In cobamide *(7)*, the hydroxyl group of cobinamide is esterified with 3'-phospho-D-ribofuranose [R = H in *(7)*]. However, corrinoids containing an *N*-glycosidylimidazole base whose second nitrogen atom is coordinated with the Co atom are also called cobamides; these cobamides may contain a benzimidazole, naphthimidazole, imidazole, or purine base. When the base is 5,6-dimethylbenzimidazole, the compound is called cobalamin *(8)*. Other ligands on the Co may be water or anions: *e.g.* in cyano-5,6-dimethylbenzimidazolylcobamide [cyanocobalamin *(8)*, L = $CN^{\ominus}$], hydroxo(aquo)adenylcobamide *(7)* [L = $HO^{\ominus}$ or H_2O, OR = adenine bound to N-7], diaquocobinamide, and monocyanomonoaquocobyric acid [10a].

(3), R = R' = OH
(4), R = NH_2, R' = OH
(5), R = OH,

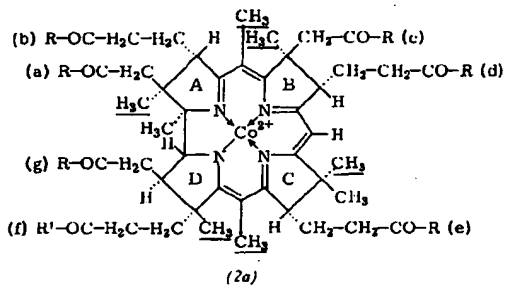
R' = –NH–CH_2–C(H)(OH)–CH_3

(6), R = NH_2,

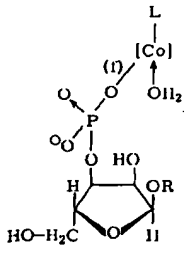
R' = –NH–CH_2–C(H)(OH)–CH_3

(9), R = NH_2,

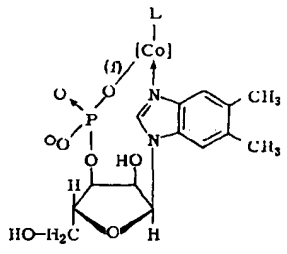
R' = –NH–CH_2–C(H)(OH)–CH_2–O–P(=O)(OH)–O



(2a)



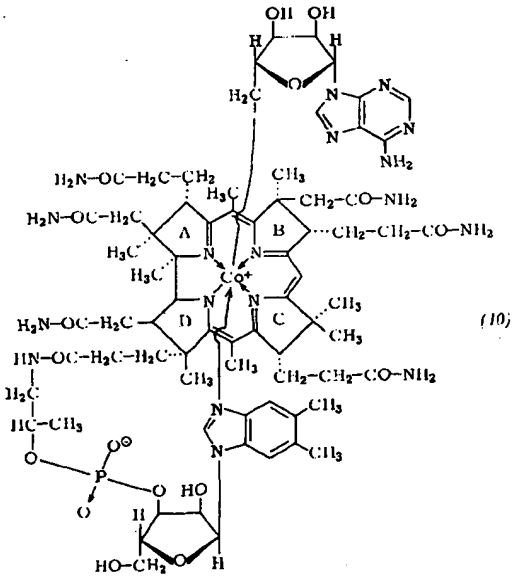
(7)



(8)

The basis for characterization and classification of corrinoids is the presence (or absence) of a hetero base which is capable of coordination. When such a base is present, the corrinoids are said to be complete, in its absence, they are said to be incomplete [11]. The two groups have significantly different physicochemical properties [11].

The coenzyme forms of the corrinoids do not contain water or anion ligands, but instead have a 5'-deoxyadenosyl residue. Thus, the simplest designation for the vitamin B_{12} coenzyme *(10)* is *Co*-(5'-β-deoxyadenosyl)-cobalamin.



(10)

[*] In this article, the designation [Co] will be used henceforth to indicate the ring system *(2a)*. In some cases with substituents, as in *(3)* to *(6)*, the meaning of [Co] is evident from the text.

B. Natural Corrinoids and Their Biogenetic Relationships [12]

So far, no naturally occurring, cobalt-free corrinoids have been isolated. The corrin ring is apparently biosynthetized in the same manner as the porphyrin ring. Thus, the explanations for the "reverse" order of acetic and propionic acid residues on ring C of the porphyrins [13, 14] should also hold for the corrinoids. The

[10a] Designations such as benzimidazolyl- or adenylcobamide cyanide are considered unconventional, at least in German nomenclature, since the substitution involves an *N*-glycosidic linkage. Furthermore, it is customary to use the prefix (*e.g.* "cyano-" and not "cyanide") in the chemistry of inorganic complex ions functioning as ligands. See *H. Remy*, Angew. Chem. *71*, 515 (1959).

[11] *K. Bernhauer* and *W. Friedrich*, Angew. Chem. *66*, 776 (1954).

[12] *K. Bernhauer, O. Müller*, and *F. Wagner* in [6], p. 37.

[13] *K. D. Gibson, M. Matthew, A. Neuburger*, and *G. T. Tait*, Nature (London) *192*, 204 (1961).

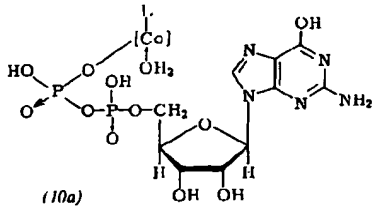
[14] *J. H. Mathewson* and *A. H. Corwin*, J. Amer. chem. Soc. *83*, 135 (1961).

additional methyl groups of the corrinoids are introduced by direct *C*-methylation. However, it is not yet known at which point of the biosynthesis this happens. It is also unclear when rings A and D are interlinked, when the Co atom is introduced, and when the acetic acid group originally probably present at C-12 is decarboxylated [14a, b]. The first part of the biosynthesis of corrinoids can be considered complete with the formation of cobyrinic acid *(3)*, even though this compound has not yet been found in nature.

The carboxyl groups a to e and g in *(3)* are amidated stepwise, and the f-carboxyl group is connected to D-(−)-1-aminopropan-2-ol, which is produced by decarboxylation of L-threonine. The intermediates produced in these reactions are cobyric acid *(4)*, several partly amidated cobinic acids *(5)*, and incompletely amidated cobyric acids. Experiments *in vivo* and *in vitro* with *P. shermanii* seem to indicate that cobyrinic acid *(3)* and its monoamide are not natural intermediates [14c]. Complete amidation leads to cobinamide *(6)*, which is the most ubiquitous intermediate in the biosynthesis of cobamides.

Occasionally a nucleotide or a part of a nucleotide is connected with a partially amidated cobinic acid, as shown by the appearance of mono-, di-, and tricarboxylic acids of cobalamin in cultures of *Propionibacterium shermanii*. It is probable that the carboxyl group e is the last to be amidated.

Cobinamide *(6)* is not directly combined with nucleotides, *e.g.* with α-ribazole phosphate at the formation of vitamin B_{12}. However, α-ribazole [14d] is incorporated directly at its biosynthesis [15] and was also found in cultures of *P. shermanii* [16]. In *Nocardia rugosa*, both cobinamide phosphate *(9)* and cobinamide pyrophosphate-guanosine *(10a)* are used for the biosynthesis of cobamides. It cannot yet be decided whether the pyrophosphate is the immediate precursor of the cobamides. For example, in experiments comparing the effect of acetone powders of *P. shermanii* on *(6)*, *(9)*, or *(10a)*,



(10a)

with 5,6-dimethylbenzimidazole, α-ribazole, or α-ribazole phosphate added, the highest conversion was obtained with the combination of *(9)* and α-ribazole [17].

Another possibility is that the initial product is cobamide *(7)*, R = H, and that its synthesis is followed by attachment of a hetero-base to the Co atom, the final step being the combination of this base with the ribose moiety via a glycosidic linkage. Several findings support this pathway. A final decision as to the mechanism will be afforded by the use of labelled substrates, which are now accessible by chemical synthesis (see below). It is quite possible that the incorporation of the nucleotide moiety follows a different pathway in the benzimidazole and purine series; it may also depend on the type of microorganism used.

It is the bases of the nucleotide moiety which account for the great variety of the cobamides, as well as for their differing physicochemical and biological properties. The strength of the bond between the bases and the Co atom decreases from a high value in 5,6-dimethylbenzimidazole and linear naphthimidazole to intermediate values in other benzimidazoles and imidazoles and a low value in the purines, thus roughly paralleling the biological activity of the respective cobamides [4]. Which base can be incorporated depends on its structure and on the type of microorganism involved [18–20]. A plausible picture of the biogenesis of benzimidazole and naphthimidazole bases emerges when it is assumed that this biosynthesis proceeds analogously to the synthesis of the benzenoid ring in riboflavin [12]. It is remarkable that under anaerobic conditions, *P. shermanii* synthetizes almost solely cobinamide *(6)*. However, in the presence of small amounts of oxygen, the same organism synthetizes 5,6-dimethylbenzimidazole and thus cobalamin *(8)* [21]. The bases of the purine series probably originate as 9-β-nucleotides, which are then hydrolysed to the free bases so that these can be incorporated into the cobamide molecule by a 7-α-glycosidic linkage.

C. Syntheses in the Vitamin B_{12} Field [22]

Recent studies in this field were aimed at the synthesis of the macrocyclic corrin system and at partial syntheses of corrinoids. The object was to obtain intermediates of the biosynthesis of vitamin B_{12}, as well as compounds with new biological properties.

I. The Corrin Ring

Several routes have been followed for the synthesis of corrin and its derivatives. *Todd* and coworkers [23, 24] studied the reactions of Δ^1-pyrroline-1-oxides, which yield dipyrrolinylmethane derivatives by condensation with the activated methyl group of 2-methyl-Δ^1-pyrroline-1-oxide. In the presence of strong bases, the Δ^1-pyrroline-1-oxides dimerize to 2,2′-dipyrrolidinyl derivatives. So far no one has reported the combining of pairs of the dipyrrolinylmethane or the 2,2′-dipyrrolidinyl derivatives or the introduction of the six adjoining and three isolated asymmetric centers into the corrin system.

[14a] *R. C. Bray* and *D. Shemin*, J. biol. Chemistry *238*, 1501 (1963).

[14b] *D. Shemin* and *R. C. Bray* in [7a].

[14c] *K. Bernhauer*, *P. Rietz*, and *F. Wagner*, unpublished results.

[14d] α-Ribazole = 5,6-Dimethylbenzimidazole-1-α-D-ribofuranoside.

[15] *P. Barbieri*, *G. Boretti*, *A. Di Marco*, *A. Migliacci*, and *C. Spalla*, Biochem. biophysica Acta *57*, 599 (1962).

[16] *H. S. Friedmann* and *D. L. Harris*, Biochem. biophysic. Res. Commun. *8*, 164 (1962).

[17] *F. Wagner*, unpublished work, Stuttgart 1962.

[18] *S. K. Kon* and *J. Pawelkiewicz:* Fourth International Congress of Biochemistry. Pergamon Press, London 1959, Vol. XI, p. 115.

[19] *D. Perlman*, Adv. appl. Microbiol. *1*, 87 (1959).

[20] *D. Perlman*, *J. M. Barrett*, and *P. W. Jackson* in [6], p. 58.

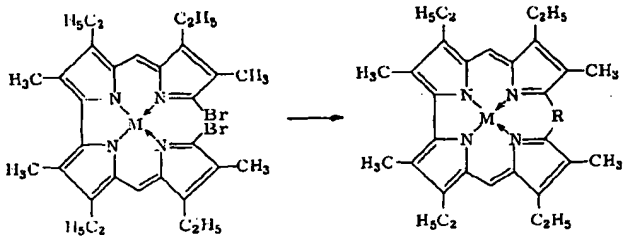
[21] US.-Pat. 2951017 (Aug. 30th, 1960), inventors: *J. D. Speedie* and *G. W. Hull*.

[22] For a review of previous work, see [5].

[23] For a review, see *A. W. Johnson* in [6], p. 1.

[24] *V. M. Clark*, Angew. Chem. *74*, 881 (1962).

Johnson and coworkers [23] synthetized pentadehydrocorrin derivatives. Even though macrocyclic compounds of structure *(11a)* were prepared from *(11)* in the presence of palladium or copper salts, these compounds



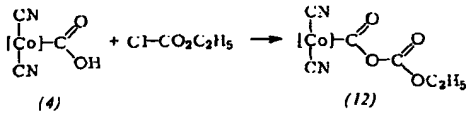
(11) *(11a)*

R = O, NH, N–CH_3, N–NH_2, S

contain an oxygen, nitrogen, or sulfur bridge between rings B and C. However, it was impossible as yet to introduce a methine bridge [24a]. The stereoselective synthesis of rings A and D of corrin is being attempted [24b].

II. Partial Synthesis of Corrinoids

Almost all partial syntheses start with cobyric acid *(4)*. The latter was isolated from digested sewage sludge [25] and obtained in crystalline form [26]; it also appears as an intermediate in the biosynthesis of vitamin B_{12} by *P. shermanii* [27] and a *N. rugosa* mutant [28]. For purely chemical syntheses [29], the carboxyl group of cobyric acid is activated by treatment with ethyl



(4) *(12)*

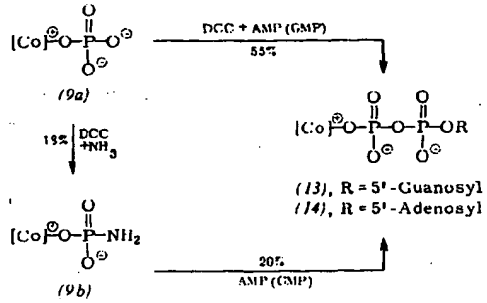
chloroformate in anhydrous dimethylformamide in the presence of triethylamine to give the mixed anhydride *(12)*, which is treated with a nucleophilic reagent without preliminary isolation. This procedure, well known in peptide chemistry, gives the best yields and is superior to the carbodiimide method [30].

1. *Incomplete Corrinoids*

Treatment of *(12)* with D-(−)-1-aminopropan-2-ol yields natural cobinamide [30]. This reaction also serves as a proof of the structure of cobyric acid *(4)*. Similarly, reactions of *(12)* with other alkanolamines yield a series of cobinamide analogues [31, 32], some of which are very strong competitive antagonists of cobinamide in *Escherichia coli* 113-3 (see below).

Reactions of *(12)* with α-amino-β-hydroxycarboxylic acids (*e.g.* serine or threonine) yield the corresponding cobinamide carboxylic acids [33]; with α-amino-β-hydroxycarboxylic acids with phosphorylated hydroxyl groups, the phosphoric esters are obtained [34]. These compounds are not metabolized by *P. shermanii* and therefore cannot be biosynthetic intermediates [34].

Cobinamide phosphate and *P*(1)-cobinamide-*P*(2)-guanosine-5′-pyrophosphate were isolated during the study of the biosynthesis of vitamin B_{12} [35]. Synthesis of these compounds from cobyric acid *(4)* proved their structure. Thus DL-cobinamide phosphate *(9a)* [*] [36] was obtained in good yield by treatment of *(12)* with DL-1-amino-2-propyl phosphate, and was in turn converted into DL-cobinamide phosphoamide *(9b)* by treatment with ammonia and *N,N′*-dicyclohexylcarbodiimide (DCC) [37]. Both of these substances can be used for the synthesis of *P*(1)-DL-cobinamide-*P*(2)-guanosine-5′-pyrophosphate *(13)* and *P*(1)-DL-cobinamide-*P*(2)-adenosine-5′-pyrophosphate *(14)*. The reaction of DL-cobinamide phosphate with adenosine-5′-phosphate



(9a) *(9b)*

(13), R = 5′-Guanosyl
(14), R = 5′-Adenosyl

(AMP) or with guanosine-5′-phosphate (GMP) in the presence of DCC does – contrary to expectation [38, 39] – not yield symmetric DL-cobinamide pyrophosphate [37] since DL-cobinamide phosphate is dipolar and ionic. Recently, natural cobinamide *(6)* was phosphorylated directly by condensing it with β-cyanoethyl phosphate and DCC in anhydrous dimethylformamide/pyridine to yield β-cyanoethylcobinamide phosphate, which gave cobinamide phosphate *(9)* upon alkaline hydrolysis [40].

[24a] *A. W. Johnson, J. T. Kay,* and *R. Rodrigo,* J. chem. Soc. (London) *1963*, 2326.

[24b] *R. B. Woodward,* Lecture in Basel (Switzerland), June 1963; Abstract: Angew. Chem. *75*, 871 (1963).

[25] *K. Bernhauer, H. Dellweg, W. Friedrich, G. Gruss, F. Wagner,* and *P. Zeller,* Helv. chim. Acta *43*, 693 (1960).

[26] *K. Bernhauer, F. Wagner,* and *D. Wahl,* Biochem. Z. *334*, 279 (1961).

[27] *K. Bernhauer, E. Becher, G. Gross,* and *G. Wilharm,* Biochem. Z. *332*, 562 (1960).

[28] *A. Di Marco, M. P. Marnati, A. Miggliacci, A. Rusconi,* and *C. Spalla* in [6], p. 69.

[29] *K. Bernhauer* and *F. Wagner* in [6], p. 28.

[30] *K. Bernhauer, F. Wagner,* and *P. Zeller,* Helv. chim. Acta *43*, 696 (1960).

[31] *K. Bernhauer* and *F. Wagner,* Hoppe-Seylers Z. physiol. Chem. *322*, 184 (1960).

[32] *K. Bernhauer, F. Wagner, D. Wahl,* and *D. Glatzle,* unpublished results.

[33] *K. Bernhauer* and *F. Wagner,* Hoppe-Seylers Z. physiol. Chem. *332*, 194 (1960).

[34] *K. Bernhauer* and *F. Wagner,* Biochem. Z. *335*, 325 (1962).

[35] See review [12].

[36] *K. Bernhauer, F. Wagner, H. Dellweg,* and *P. Zeller,* Helv. chim. Acta *43*, 700 (1960).

[37] *K. Bernhauer* and *F. Wagner,* Biochem. Z. *335*, 453 (1962).

[*] DL-Cobinamide phosphate is the short designation for cobyryl-(DL-2-hydroxypropyl)amide. Cobinamide denotes the natural product.

[38] *H. G. Khorana,* Fed. Proc. *19*, 931 (1960).

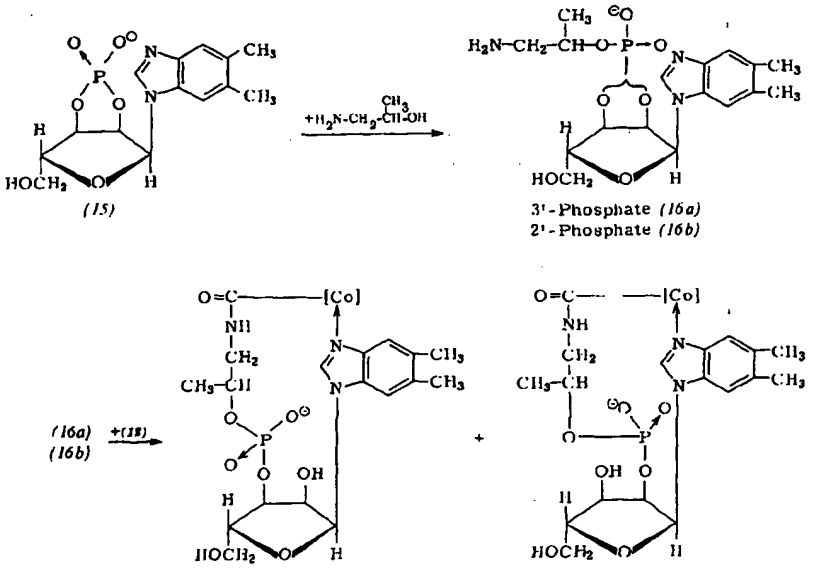
[39] *F. Cramer,* Angew. Chem. *72*, 236 (1960).

[40] *F. Wagner,* Biochem. Z. *336*, 99 (1962).

These methods permitted preparation of other phosphorylated cobinamide derivatives, including radioactively labelled preparations. These are valuable for elucidating the mode of biosynthesis of the nucleotide moiety of the complete cobamides.

2. *Complete Corrinoids*

Friedrich et al. [41] were the first to synthetize cobalamin *(8)*, starting from cobyric acid *(4)*: α-ribazole phosphate [(1-α-D-ribofuranosyl-5,6-dimethylbenzimidazolyl)-3′-dihydrogen phosphate] was converted into α-ribazole-2′,3′-cyclophosphate *(15)* by reaction with dicyclohexylcarbodiimide. Compound *(15)* was then



reated with a large excess of an alkanolamine (*e.g.* D-1-aminopropan-2-ol in the synthesis of cobalamin) in the presence of sodium butoxide. This gave a 70 % yield of a mixture of equal parts of the 3′- and 2′-nucleotide esters *(16a)* and *(16b)*. The next reaction, in which the mixture was treated with one mole of *(12)* in anhydrous dimethylformamide, was complete at 0 °C within a few seconds giving a 60–70 % yield of the complete corrinoid [43].

This same method [41] was used to prepare 5-methoxybenzimidazolylcobamide and 2-methyladenylcobamide [42]. The complete cobamides obtained by treatment of the 3′-(D-1-amino-2-propyl)nucleotide ester proved to be identical with the corresponding natural products. This is a further proof for the structure determined analytically.

The mixture of isomers *(16a)* and *(16b)* can be separated by chromatography on DEAE-cellulose or on paper. Alternatively, the mixture is treated with *(11)* and the 3′- and 2′-vitamin B_{12} derivatives are separated by paper electrophoresis at pH 2.7 [43].

This synthetic pathway permits the preparation of numerous vitamin B_{12} derivatives [43] in which the D-1-aminopropan-2-ol group of cobalamin is replaced by other alkanolamines (see below). These products, which are not found in nature, may help in the elucidation of the biochemical function of the D-1-aminopropan-2-ol group. Some of these compounds were shown to be exceptionally strong competitive antagonists of cobalamin (see below).

In addition to the purely synthetic 2′-isomer of cobalamin [41], the 5′-isomer of cobalamin was recently prepared [40] by condensation of cobinamide phosphate and 2′,3′-isopropylidene-α-ribazole with dicyclohexylcarbodiimide, followed by removal of the isopropylidene group. The 5′-isomer was also synthetized by condensation of α-ribazole-5′-phosphate with 1-(benzyloxycarbonylamino)-2-propanol in the presence of dicyclohexylcarbodiimide, hydrogenolytic cleavage of the carbobenzoxy group and reaction of the diester so obtained with *(12)* [43a]. Direct phosphorylation of cobalamin by the method described for cobinamide yields cobalamin-5′-phosphate [40].

D. Coenzyme Forms of the Corrinoids

The commercial cyano form of cobalamin is an artefact. It is obtained by the action of cyanide ions on natural precursors of the vitamin during the isolation of the latter. Only the hydroxo (aquo) form can be isolated from natural substrates, provided cyanide ions are rigidly excluded. Because of its good depot properties, it is preferred over the cyano form [44–45], and it is in that form that vitamin B_{12} is commonly used. However, the naturally occurring vitamin actually exists in the coenzyme form discovered several years ago.

[41] *W. Friedrich, G. Gross, K. Bernhauer*, and *P. Zeller*, Helv. chim. Acta *43*, 704 (1960).

[42] *W. Friedrich* and *H. C. Heinrich*, Biochem. Z. *333*, 550 (1961).

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[44] *E. E. Gabbe* and *H. C. Heinrich* in [6], p. 116.

[44a] *G. B. J. Glass, H. R. Skeggs, D. H. Lee, E. L. Jones*, and *W. W. Hardy* in [6], p. 673.

[45] *G. B. J. Glass, D. H. Lee, H. R. Skeggs*, and *J. L. Stanley*, Fed. Proc. *21*, 471 (1962).

I. Occurrence and Isolation of the Coenzymes [46]

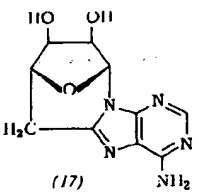
The corrinoid coenzymes were discovered by *Barker* et al. [47] during a study of the enzymatic conversion of glutamic acid into β-methylaspartic acid. The enzyme is light-sensitive and is deactivated by daylight or by cyanide ions. The coenzyme forms of 5,6-dimethylbenzimidazolylcobamide, benzimidazolylcobamide, and adenylcobamide were the first to be isolated [47]. Later, coenzyme forms of cobinamide [48,49], cobinamide-pyrophosphate-guanosine [48], cobyric acid [50], and several biosynthetic cobamides [51,52] were isolated from microorganisms. Vitamin B_{12} also exists in its coenzyme form *(10)* in the liver of humans, sheep, rabbits, and chickens [53].

To isolate the coenzyme forms, bacteria are extracted with boiling 70–80 % ethanol or a neutral aqueous buffer [46], or with 60 % aqueous acetone at room temperature [54]. Acetone powders of bacteria are extracted with water [48]. After concentration, the solutions are extracted with mixtures of phenol and chloroform or *o*-dichlorobenzene (40:60 w/w). The organic phase is washed several times with water, treated with chloroform and n-butanol. The corrinoid coenzymes can then be extracted with water. Residual phenol is removed with chloroform. The aqueous solution is concentrated *in vacuo*, and usually contains mixtures of the coenzymes. These can be separated by column chromatography on cellulose, by paper electrophoresis, or by ion exchange [46, 54].

II. Properties and Degradation of the Coenzymes

The extreme sensitivity to light of the isolated coenzymes and their peculiar cleavage by cyanide are their most remarkable properties. The coordinate bond between cobalt and the imidazole nitrogen is ruptured at pH < 6 if the hetero base is a purine and at pH < 2.5 if the coenzyme contains benzimidazole [46].

It is concluded from the valence of the cobalt in the cobalamin coenzyme (see below) and from the appearance of a reduced form of cobalamin (B_{12r}) after exposure to light in the absence of oxygen [55, 56] that the ligand containing adenine is split off as a radical. In the absence of oxygen, the radical is stabilized by formation of the cyclic nucleoside *(17)* [57, 57a]. In air, the 8,5′-cyclic adenosine is only partially formed; some of the radicals react with oxygen to form adenosine-5′-aldehyde *(18)* [58] or adenosine-5′-carboxylic acid *(19)* [59]. In air, vitamin B_{12r} is oxidized within a few seconds to hydroxo (aquo) cobalamin. The corrinoid coenzymes are much more stable when bound to proteins than in their free form.



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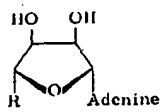
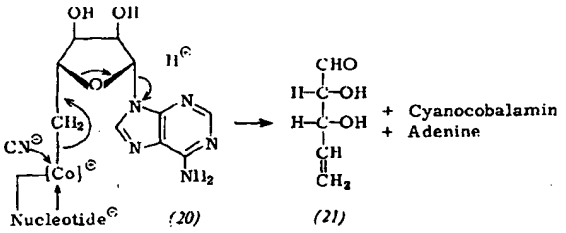


(18), R = CHO
(19), R = COOH

Cyanide converts the cobalamin coenzyme *(20)* [= *(10)*] into cyanocobalamin [46, 59, 59a] by splitting off adenine and *erythro*-3,4-dihydroxy-1-penten-5-al *(21)*. This reaction is not affected by atmospheric oxygen [55].



(20) *(21)*

The other corrinoid coenzymes are cleaved by light and cyanide in the same manner as the cobalamin coenzyme, but at considerably different rates [60].

The action of iodine on the cobalamin coenzyme in aqueous solution results in the formation of iodinecobalamin and 5′-iodo-5′-deoxyadenosine [54].

III. Structure of the Coenzymes

The structure of the cobalamin coenzyme *(10)* was first elucidated by means of X-ray analysis [61] and was then confirmed by partial synthesis (see below).

In the cobalamin coenzyme, the 5′-deoxyadenosyl residue assumes the sixth coordination position, which is occupied by cyanide in vitamin B_{12}. It is connected to the Co atom by a covalent bond at C-5′. X-ray diffraction studies on the cobalamin coenzyme have shown that, like vitamin B_{12}, it contains Co^{3+} [62]. This is in agreement with its electrophoretic behavior. The presence of Co^{3+} is further indicated by the electron spin resonance spectrum of the cobalamin coenzyme [62a].

[46] *H. A. Barker* in [6], p. 82.

[47] *H. A. Barker, H. Weissbach*, and *R. D. Smyth*, Proc. nat. Acad. Sci. USA *44*, 1093 (1958).

[48] *J. Pawelkiewicz, B. Bartosiński*, and *W. Walerych*, Bull. Acad. polon Sci., Sér. Sci. biol. *8*, 123 (1960).

[49] *K. Bernhauer, P. Gaiser, O. Müller*, and *O. Wagner*, Biochem. Z. *333*, 106 (1960).

[50] *A. Migliacci* and *A. Rusconi*, Biochim. biophysica Acta *50*, 370 (1961).

[51] *K. Bernhauer, O. Müller*, and *G. Müller*, Biochem. Z. *335*, 37 (1961).

[52] *J. I. Toohey, D. Perlman*, and *H. A. Barker*, J. biol. Chemistry *236*, 2119 (1961).

[53] *J.I.Toohey* and *H.A.Barker*, J. biol. Chemistry *236*, 560 (1961).

[54] *K. Bernhauer, P. Gaiser, E. Irion, G. Müller, O. Müller*, and *O. Wagner*, unpublished results.

[55] *K. Bernhauer* and *O. Müller*, Biochem. Z. *334*, 199 (1961).

[56] *R. O. Brady* and *H. A. Barker*, Biochem. biophysic. Res. Commun. *4*, 373 (1961).

[57] *H.P.C. Hogenkamp* and *H.A. Barker*, Fed. Proc. *21*, 470 (1962).

[57a] *H. P. C. Hogenkamp*, J. biol. Chemistry *238*, 477 (1963).

[58] *H. P. C. Hogenkamp, J. N. Ladd*, and *H. A. Barker*, J. biol. Chemistry *237*, 1950 (1962).

[59] *A. W. Johnson* and *N. Shaw*, Proc. chem. Soc. (London) *1961*, 447.

[59a] *A. W. Johnson* and *N. Shaw*, J. chem. Soc. (London) *1962*, 4608.

[60] *O. Müller* and *G. Müller*, Biochem. Z. *336*, 299 (1962).

[61] *P. G. Lenhert* and *D. Crowfoot-Hodgkin*, Nature (London) *192*, 937 (1961).

[62] *D. Heintz*, Diploma Thesis, Universität München 1962.

[62a] *H. P. C. Hogenkamp, H. A. Barker*, and *H. S. Mason*, Arch. Biochem. Biophysics *100*, 353 (1963).

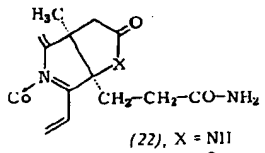
Thus, magnetochemical results which were obtained with aqueous solutions of the cobalamin and cobinamide coenzymes and which indicate Co^{2+} [63–65] are probably due to other phenomena.

The cobinamide coenzyme contains a hydroxo (aquo) group instead of the nucleotide. The cobinamide coenzyme obtained by degradation of cobalamin coenzyme *(10)* with cerium(III) hydroxide [66] is identical with the natural product; this proves the position of the nucleoside.

At first it was not clear whether the corrin ring of the coenzyme forms had the usual six double bonds. The bond system and the number of hydrogen atoms in the corrin skeleton of the cobalamin coenzyme cannot be determined exactly by X-ray analysis [61]. The partial synthesis of the coenzyme forms involves reduction of the Co atom and thus, does not yield any definite conclusions about the structure of the corrin ring.

The synthesis of *Co*-methylcobalamin in water-containing tritium leads to a radioactive product [67] with tritium in the methyl group bound to the cobalt. This was shown [67a] by aerobic photolysis and trapping of the produced formaldehyde in the form of its adduct with dimedone. This results seems to indicate that the conjugated system of the corrin ring in the coenzyme form is identical with that of the cyano forms [67a].

It was shown, however [67], that the coenzyme forms do not undergo the same reactions as cyano- or hydroxocobalamin do on account of activation of C-8 (CN double bond in the allylic position). Thus, in alkaline solution, cyanocobalamin *(8)*, L = CN, is dehydrogenated by atmospheric oxygen to the lactam *(22)* [68] while cobalamin coenzyme, cobalamin sulfonate or *Co*-methylcobalamin are not dehydrogenated under the same conditions. Equimolar

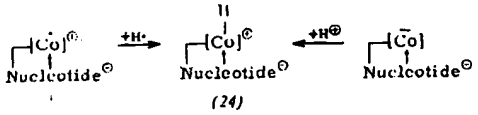


(22), X = NH
(23), X = O

quantities of chloramine-T or bromine water oxidize cyanocobalamin to the lactone *(23)*. Halogenation occurs only when chloramine-T or bromine water is present in excess. On the other hand, the coenzyme forms are not oxidized by one mole of chloramine-T, but are converted into a uniform monochloro derivative [67]. The first equivalent of bromine water or *N*-bromosuccinimide has an analogous effect [68a].

IV. Partial Chemical Syntheses of Corrinoid Coenzymes and Their Analogues

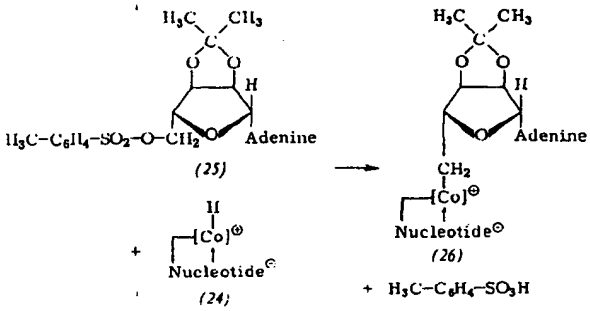
When vitamin B_{12} is reduced with zinc in NH_4Cl [69], NaOH, or acetic acid solution, yellow B_{12r} is initially formed, provided oxygen is rigorously excluded. After prolonged reaction times, the reduction proceeds further, yielding a light blue to green product. This product can also be obtained with other reducing agents, *e.g.* chromium(II) salts or sodium borohydride [70,71]. Cobalamin reduced in this way reacts with diazomethane to yield *Co*-methylcobalamin and is thus a cobalt hydride *(24)* [60]. It is formed in the reaction of the Co^{2+}-complex with nascent hydrogen, or from the intermediate Co^{+}-complex by addition of a proton. The addition reactions of the reduced product also seem to indicate a cobalt-hydrogen bond.



(24)

Provided oxygen is absent, cobalamin hydride is stable in aqueous solution. In air, it is converted within a few seconds to hydroxo(aquo)cobalamin.

Hydrides of other corrinoids, such as benzimidazolylcobamide, adenylcobamide, and cobinamide, can be obtained in a similar manner. The reduction to the hydride causes the cobalt atoms of the corrinoids to become nucleophilic and thus to react with compounds having an electrophilic center.



(25) *(26)*

The synthesis of the cobalamin coenzyme proceeds by reaction of cobalamin hydride *(24)* with 2′,3′-isopropylidene-5′-tosyladenosine *(25)* to yield *(26)*, followed by removal of the isopropylidene group with dilute acid [60, 72,73,73a]. Similarly, reaction of cobalamin hydride and 2′,3′-isopropylidene-5′-tosylinosine yields the hypoxanthine analogue [72,74] of the cobalamin coenzyme. This analogue had been previously prepared by deamination of the cobalamin coenzyme [75]. The uridine [72] and guanosine [74] analogues of cobalamin coenzyme have also been obtained. It is noteworthy that synthetic cobinamide coenzyme is identical with the natural product. This implies that the hydrogen atom attached to the Co in the hydrides is always on the same side of the molecule.

[63] *A. W. Johnson* and *N. Shaw*, Proc. chem. Soc. (London) *1960*, 420.

[64] *L. Nowicki* and *J. Pawelkiewicz*, Bull. Acad. pol. Sci., Sér. Sci. biol. *8*, 433 (1960).

[65] *K. Bernhauer, P. Gaiser, O. Müller, E. Müller*, and *F. Günter*, Biochem. Z. *333*, 560 (1961).

[66] *K. Bernhauer* and *O. Müller*, Biochem. Z. *335*, 44 (1961).

[67] *F. Wagner* and *P. Renz*, Tetrahedron Letters *1963*, 259.

[67a] *F. Wagner* and *K. Bernhauer* in [7a].

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[68a] *F. Wagner* and *V. Koppenhagen*, unpublished results.

[69] *O. Schindler*, Helv. chim. Acta *34*, 1356 (1951).

[70] *R. N. Boos, J. E. Can*, and *J. B. Conn*, Science (Washington) *117*, 603 (1953).

[71] *F. P. Siegel*, Ph. D. Thesis, University of Illinois, USA, *1955*.

[72] *E. L. Smith, L. Merwin, A. W. Johnson*, and *N. Shaw*, Nature (London) *194*, 1175 (1962).

[73] *K. Bernhauer, O. Müller*, and *G. Müller*, Biochem. Z. *336*, 102 (1962).

[73a] *A. W. Johnson, N. Shaw*, and *E. L. Smith*, J. chem. Soc. (London) *1963*, 4146.

[74] *O. Müller* and *G. Müller*, Biochem. Z. *336*, 299 (1962).

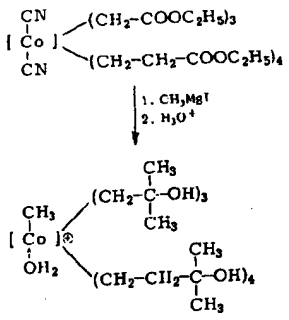
[75] *O. Müller* and *G. Müller*, Biochem. Z. *335*, 340 (1962).

V. Other Corrinoids with Cobalt-Carbon Bonds

Alkylated cobalamin compounds are obtained by treatment of cobalamin hydride with alkyl halides, dialkyl sulfates, or *p*-toluenesulfonic esters [60,72–74]. At room temperature, the reactions are finished within a few minutes. Sulfonium compounds such as methylmethionine or *S*-adenosylmethionine can also be used to prepare *Co*-methylcobalamin [74,75a]. In addition, acetylenic and olefinic compounds add onto cobalamin hydride. For example, reaction with acrylic acid yields *Co*-β-carboxyethylcobalamin [73a,75b], while that with tetrahydrofuran yields *Co*-δ-hydroxybutylcobalamin [74]. As expected from their structures, the properties of the *Co*-alkylcorrinoids resemble those of the corrinoid coenzymes. In air, they are photolysed to the corresponding hydroxo(aquo)corrinoids. The ultraviolet absorption spectra of the *Co*-alkylcorrinoids are also very similar to those of the corresponding corrinoid coenzymes. However, in contrast to the coenzymes, the *Co*-alkylcorrinoids are not cleaved by cyanide.

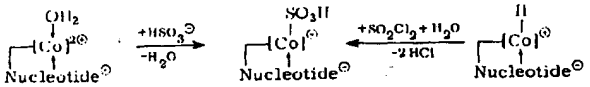
The corrinoid hydrides also react with acylating agents such as acid anhydrides and acyl halides, producing acyl *Co*-derivatives. These are also sensitive to light and cyanide and, in addition, to alkali [60]. It is noteworthy that the ultraviolet spectrum of *Co*-ethoxycarbonylcobalamin, which is obtained by the reaction of cobalamin hydride with ethyl chloroformate, is much more similar to the ultraviolet spectrum of cyanocobalamin than to that of the coenzyme forms [74].

But even Cyano- or hydroxocorrinoids with Co^{3+} as central atom can be directly converted into coenzyme-like derivatives, when compounds are used which are soluble in inert solvents and which can therefore be reacted with Grignard reagents or lithium alkyls. For example, treatment of heptaethyl cobyrinate with excess methylmagnesium iodide in tetrahydrofuran/ether and decomposition of the reaction product with diluted acetic acid yields a *Co*-methyl derivative of the corresponding tertiary alcohol [67a].



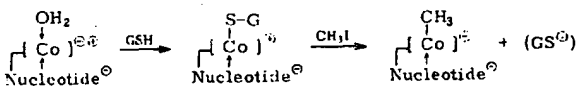
VI. Corrinoids with Cobalt-Sulfur Bonds

The action of sulfite or sulfurous acid on cyano- or hydroxo(aquo)corrinoids yields substances which are very similar to the corrinoid coenzymes [76–80]. These are again light-sensitive, have similar ultraviolet spectra, and are converted by cyanide into cyanocorrinoids. The derivatives of cobalamin and cobinamide were obtained in crystalline form [76,79,79a]. These same produtcts are also produced on treatment of cobalamin hydride or cobinamide hydride with sulfuryl chloride [79]. These reactions, as well as the appropriate infrared spectra, show that these corrinoids contain a cobalt-sulfur bond. Until the nomenclature of this new class of compounds is fixed, we will designate these substances respectively



as cobalamin *Co*-sulfonate and cobinamide *Co*-sulfonate. *Co*-*p*-Toluenesulfonyl- and *Co*-benzenesulfonylcobinamides were prepared in the same manner. However, these compounds are not light-sensitive [79].

Treatment of aquocobalamin with glutathione (GSH) yields *Co*-(*S*-glutathionyl)cobalamin as intermediate which reacts with electrophilic agents, *e.g.* with methyl iodide to give *Co*-methylcobalamin [67a].



VII. Biosynthesis of the Corrinoid Coenzymes

All the corrinoids occurring in nature apparently exist in the coenzyme form. This form presumably arises soon after the synthesis of the corrin ring and the incorporation of the Co atom, perhaps at the stage of a pentacarboxylic acid *(3)*, which, just like other related polycarboxylic acids, can be converted enzymatically into its coenzyme form [81]. Further biogenesis to the complete cobamides probably takes place at the coenzyme level. Alternatively, it may involve forms having the essential characteristics of the coenzyme structure (see below).

The biosynthesis of the 5'-deoxyadenosyl moiety and its binding to the Co atom was studied mainly with enzymes obtained from microorganisms. These studies were first conducted with acetone powders [82–84], later with extracts of bacteria [85–89], and finally with

[75a] *W. Friedrich* and *E. Königk*, Biochem. Z. *336*, 444 (1962).
[75b] *E. L. Smith* and *L. Merwyn*, Biochem. J. *86*, 2P (1963).
[76] *K. Bernhauer, O. Müller*, and *O. Wagner* in [6], p. 110.
[77] *K. Bernhauer, P. Renz*, and *F. Wagner*, Biochem. Z. *335*, 443 (1962).
[78] *Ph. George, D. H. Irvine*, and *St. C. Glauser*, Ann. New York Acad. Sci. *88*, 393 (1960).
[79] *K. Bernhauer* and *O. Wagner*, Biochem. Z. *337*, 366 (1963).
[79a] *D. H. Dolphin, A. W. Johnson*, and *N. Shaw*, Nature (London) *199*, 170 (1963).
[80] *J. A. Hill, J. M. Pratt*, and *R. J. P. Williams*, J. theoret. Biol. *3*, 423 (1962).
[81] *K. Bernhauer, H. Beisbarth, P. Rietz*, and *F. Wagner*, unpublished results.
[82] *K. Bernhauer, P. Gaiser, O. Müller*, and *O. Wagner*, Biochem. Z. *333*, 106 (1960).
[83] *J. Pawelkiewicz, B. Bartosiński*, and *W. Walerych*, Bull. Acad. polon. Sci., Sér. Sci. biol. *8*, 123 (1960).
[84] *J. Pawelkiewicz, B. Bartosiński*, and *W. Walerych*, Acta biochim. polon. *8*, 131 (1961).
[85] *H. Weissbach, B. G. Redfield*, and *A. Peterkofsky*, J. biol. Chemistry *236*, PC40 (1961).
[86] *R. O. Brady* and *H. A. Barker*, Biochem. biophysic. Res. Commun. *4*, 464 (1961).

a purified enzyme preparation which had been enriched 337 times [90]. When this preparation is used, the synthesis of the coenzyme requires Mn^{2+}, K^+, reduced flavin-adenine dinucleotide and a sulfhydryl compound, in addition to a corrinoid and ATP. Studies with ATP labelled with radiocarbon show that this is the source of the 5'-deoxyadenosyl residue of the coenzyme [87–90]. However, the manner in which the 5'-deoxyadenosyl residue is transferred is unknown. The enzymatic conversion of cyanocobalamin into the cobalamin coenzyme is supposed to take place in one step [91]. However, by starting with cobinamide, it was possible to isolate a labile, light-sensitive, yellow, apparently reduced intermediate, with physicochemical properties reminiscent of reduced cobinamide. This product then yielded the cobinamide coenzyme in a second reaction step which took place in the presence of ATP and dihydroflavin mononucleotide ($FMNH_2$) [92,93].

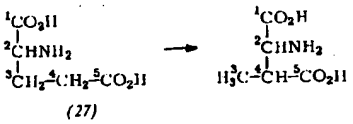
E. Enzymatic Functions of Vitamin B_{12}

I. Intramolecular Rearrangements in which Cobamide Coenzymes are Involved

The enzymatic reactions in which cobamide coenzymes participate are mainly intramolecular isomerizations.

1. *Conversion of Glutamate into Methylaspartate*

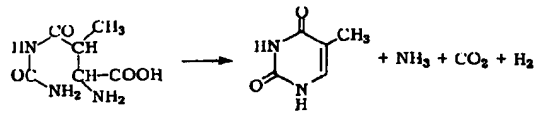
The study of this reaction in *Clostridium tetanomorphum* led to the discovery of the coenzyme forms of the cobamides [46]. The glutamate isomerase reaction can be conceived as an intramolecular reversible transfer of a glycine group from the β- to the α-carbon of the propionic acid moiety of glutamic acid *(27)*, with simultaneous shift of a hydrogen atom in the opposite direction



(27)

[46]. Only cobamide coenzymes are active in this reaction; the incomplete forms are inactive [94,94a].

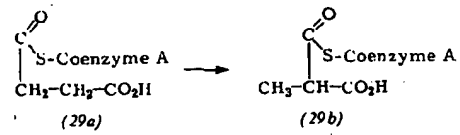
In protozoa, the carbamyl derivative *(28)* of methylaspartate is a precursor of thymine, which may explain why vitamin B_{12} is required for DNA synthesis [95]. However, this pathway of thymine biosynthesis does not apply to rats [96].



(28)

2. *Conversion of Succinyl-CoA into Methylmalonyl-CoA*

This reaction is catalysed by methylmalonyl-coenzyme-A isomerase and involves transfer of the thioester group from the β- to the α-carbon of the propionic acid moiety of the molecule [*i.e. (29a)* → *(29b)*], as was shown with labelled substrates [97,98]. When purified isomerase



(29a) *(29b)*

was used, the reaction product did not take up tritium from labelled water [99]. For the reaction mechanism, see also [99a,b].

This reaction plays an important role in the biological utilization of propionic and other fatty and amino acids [100–102b]. It is noteworthy that in human megaloblastic anemia, ten to twenty times the normal amount of methylmalonate is excreted in the urine. Thus, accumulation of methylmalonate in the tissues could be the cause of the symptoms of pernicious anemia [103].

3. *Conversion of 1,2-Diols into Deoxyaldehydes*

This intramolecular redox reaction was realized with cell-free extracts of *Aerobacter aerogenes* or *Clostridium perfringens* in the presence of some cobamide coenzymes; examples are: propane-1,2-diol → propionaldehyde and ethylene glycol → acetaldehyde [104,104a]. During this study, it was shown by experiments with heavy water that the rearrangement involves an intramolecular shift of hydrogen (as hydride ion) with simul-

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taneous displacement of an OH group [105]. Recently, acetaldol was suggested as an intermediate in the reation [106]. Extracts of lactobacillus convert glycerol to β-hydroxypropionaldehyde [106a, b].

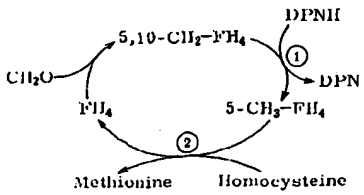
II. Degradation of Lysine to Fatty Acids and Ammonia

$$H_2N-(CH_2)_4-CH(NH_2)-CO_2H \longrightarrow H_3C-(CH_2)_2-CO_2H + H_3C-CO_2H + 2NH_3$$

To accomplish this reaction, which is effected by *Clostridia*, cell preparations that have been aged or treated with activated charcoal require pyruvate, diphosphopyridine nucleotide, Fe^{2+}, acetyl coenzyme A, and cobalamin coenzyme [107, 107a].

III. The Role of Vitamin B_{12} in Methionine Synthesis

A cobalamin enzyme is involved, in addition to several other co-factors, in the transfer of a methyl group from *N*(5)-methyltetrahydrofolic acid to homocysteine [108 to 112a]. This process is part of a cycle which explains the close biochemical relationship known to exist between folic acid and vitamin B_{12} (Scheme 1).



Scheme 1. The role of vitamin B_{12} in the transfer of a methyl group in the synthesis of methionine.

(1) 5,10-methylenetetrahydrofolate reductase
(2) B_{12}-enzyme, DPNH, $FADH_2$, ATP, Mg^{2+}

It is possible that this enzymatic system is a main metabolic function of vitamin B_{12} in animal cells and perhaps even the key to various anemias, for in vitamin B_{12} deficiency, the level of methyltetrahydrofolic acid in blood rises to a value several times above the normal. Thus the synthesis of many cell components (purines, pyrimidines) may be inhibited by blockage of the folic acid cycle [112].

In methionine synthesis, the active portion of the vitamin B_{12} enzyme appears to be *Co*-methylcobalamin. If this is incubated with homocysteine and a purified apovitamin B_{12} enzyme, methionine is synthetized and the methyl group bound to cobalt is utilized [113, 113a].

Methionine can also be synthetized nonenzymatically by anaerobic photolysis of *Co*-methylcobalamin in the presence of homocysteine. Under identical conditions, homocysteine is converted by the cobalamin coenzyme into *S*-adenosylhomocysteine [114]. These photolytic reactions can be explained by a free radical mechanism.

The activity of the coenzyme forms in the above enzyme systems explains only a part of the manifold and vital functions of vitamin B_{12} [115]. Moreover, the vitamin also acts as a cofactor in the reduction of ribonucleosides to deoxyribonucleosides [116–118], in the incorporation of amino acids into protein [119, 120], and in carbohydrate and fat metabolism [121, 122].

IV. Enzymatic Synthesis of Methane

In the presence of pyruvate, an enzyme system from *Methanosarcina barkeri* converts the methyl group of *Co*-methylcobalamin stoichiometrically to methane, as was shown with labelled substrates [122a]. The same reaction occurs with extracts of *Methanobacillus omeianskii* in the presence of ATP [122b].

F. Molecular Biology of Vitamin B_{12}

The biochemical function of the various portions of the vitamin B_{12} molecule was elucidated primarily – as for other vitamins – by demonstration of the biological activity of analogues which were prepared chemically. Analogues with antagonistic activity may be suitable for the treatment of leukemia or other malignant diseases (for reviews, see [123, 124]).

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I. The Cobalt Atom and the Corrin Ring

In contrast to the porphyrins, the metal atom in vitamin B_{12} is held so tightly that it has not yet been possible to remove it without destroying the molecule. The Co atom participates in the resonance of the corrin ring system. When coordinated nucleotides are present, the Co atom is so deeply imbedded in the cobamide coenzymes that it cannot come into direct contact with substrate. Contact is probably made via a peripheral part of the molecule [46, 125].

The difference in reactivity of the two coordination positions of the Co atom in incomplete corrinoids gave the first insight into the significance of the corrin ring. Thus, X-ray analysis has shown that the cyano group in the monocyanomonochlorohexacarboxylic acid [126] or in monocyanomonoaquocobyric acid *(4)* [127] is coordinated at the same position as the nucleotide in cobalamin *(8)*. Furthermore, in dicyanocobinamide *(6)*, which has two CN groups as ligands, one CN group is bound tightly and the other loosely [77]. It is remarkable that the coenzyme form obtained by chemical synthesis from cobinamide is identical with the natural product and that no other isomer is obtained [74]. Parallel to this, *P. shermanii* converts synthetic *Co*-butylcobinamide into the same *Co*-butylcobalamin as can be synthetized directly from cobalamin [74]. Thus, hydride formation in cobinamide can take place only in the "upper" coordination position in the formulae shown here; this requirement must hold for both incomplete and complete corrinoids. It is possible that the not exact planarity of the corrin ring [126] or the *trans*-effect [128] is of importance in this connection.

II. The 1-Aminopropan-2-ol Group

Surprisingly, the 1-aminopropan-2-ol group is of special molecular-biological significance, as was shown by experiments with many analogues containing a modified alkanolamine moiety [124]. The most important representatives are shown in Table 1. The stimulation of the growth of *E. coli* 113-3 by chemically and in part biochemically synthetized analogues is similar to that by cobalamin [130] and cobinamide [32], provided the C-1 of the 1-aminoethan-2-ol group carries no substituent (Table 1). If they are not too large, substituents on C-2 do not reduce growth stimulation significantly, compared with the natural products. However, substitution on C-1 results in strong competitive antagonism. This is also true for the coenzymeforms of cobinamide analogues

Table 1. Effect of cobalamin and cobinamide analogues on *E. coli* 113-3 (tube-test experiments).

1-Aminoethan-2-ol group with substituent on		Effect on growth, compared to		Inhibition Index [129]	
C-1	C-2	Cobalamin = 100	Cobinamide = 100	Cobalamin	Cobinamide
H	H	83	71	–	–
H	CH_3 (D)	100	100	–	–
H	CH_3 (L)	60	80	–	–
H	Phenyl (DL)	0.01	0.01	17	32
H	2,CH_3	45	36	–	–
CH_3	CH_3	1	36		–
CH_3	H	0.1	0.01	6	7
C_2H_5	H	–	0.01	–	5
2 CH_3	H	0.1	0.01	3	3

[32] and of cobalamin analogues [130a]. Cobalamin antimetabolites also inhibit the growth of *Ochromonas malhamensis* and are antierythropoetic in decompensated pernicious anemia patients [130]. Cobinamide analogues which stimulate the growth of *E. coli* are converted into cobalamin analogues by *P. shermanii* in the presence of 5,6-dimethylbenzimidazole. This is not so with cobinamide antagonists [32].

III. The Carboxamide Groups

The carboxyl groups in positions a–e and g [see formula *(2a)*] must be amidated for vitamin B_{12} to be biochemically active; cobalamincarboxylic acids are inactive or antagonistic towards *E. coli* [123, 131]. The individual carboxamide groups are, however, of differing biochemical significance. The strongest antagonist to *E. coli* [123, 132] is the cobalaminmonocarboxylic acid which is the last intermediate in the biosynthetic amidation [81, 132] and is also the one which is the predominant product of mild acidic hydrolysis of cobalamin. Its carboxyl group in position e is presumed to be the one that is free [81]. Its inhibition index (see [129]) with *E. coli* is 40 [133]. The alkylamides of cobalamin which are synthetized by alkylamidation of the carboxylic acids are also antagonistic to *E. coli*. The most active representatives are the monomethylamide and the hydrazide of that monocarboxylic acid which is the main product of hydrolysis of cobalamin (inhibition index = 50) [123].

IV. The Nucleotide Moiety

Only cobamide coenzymes which contain nucleotides apparently have biochemical activity *in vivo*; the incomplete forms are only intermediates of the biosynthesis. In biological systems in which the incomplete forms are active (*e.g.* in growth tests), they are converted beforehand into complete cobamide coenzymes.

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The nature of the base in the nucleotide portion is of minor importance in bacteria, but is very important in the protozoon *O. malhamensis* and in animals. Here, only cobamides of the benzimidazole and naphthimidazole series are active. Although many cobamide analogues containing various non-naturally occurring bases have been synthetized [4, 18, 19], it was impossible to obtain a cobalamin antagonist with a good inhibition index in this way. The 3'-phosphoric acid bond in the nucleotide permits the base to coordinate with the cobalt [see formula *(8)*], so that the molecule becomes "complete" – a fact which appears to be of biochemical importance. The 2'-analogues, in which the bond between Co and the imidazole ring is weakened, are biologically less active [124].

V. The 5'-Deoxyadenosyl Group of the Coenzyme Forms

The 5'-deoxyadenosyl group is necessary for the activity of the coenzyme forms in the enzyme systems mentioned above. Its replacement by other ligands, such as 5'-deoxyinosine, 5'-deoxyuridine, or alkyl groups, leads to loss of activity or to competitive antagonism [72, 100]. However, the presence of the 5'-deoxyadenosyl group is not necessary for the biosynthesis of the vitamin B_{12} molecule, for *Co*-ethyl- and especially *Co*-butylcobinamide are converted into the *Co*-alkylcobalamines by *P. shermanii in vivo* if 5,6-dimethylbenzimidazole is present. Only afterwards they are converted into cobalamin coenzymes [74].

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New Methods of Preparative Organic Chemistry IV [*]

Cyclization of Dialdehydes with Nitromethane [1]

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Dedicated in memoriam to Hermann O. L. Fischer, whose initiative started the developments in this field

Condensation of nitromethane with suitable dialdehydes in alkaline medium provides a general method of cyclization, in which the methyl group of the nitromethane is incorporated into the ring. This method leads to 5-, 6-, and 7-membered rings and is equally applicable to aliphatic, aromatic, and sugar dialdehydes. For example, glyoxal is converted into 1,4-dideoxy-1,4-dinitro-neo-inositol, and glutaraldehyde into trans-2-nitrocyclohexane-1,3-diol, while the corresponding cyclization of xylo-trihydroxyglutaraldehyde leads to deoxynitroinositols having the scyllo, myo-1, and muco-3 configurations. – In the case of aromatic dialdehydes, the cyclization is accompanied by elimination of water. Thus, phthalaldehyde, naphthalene-2,3-dicarboxaldehyde, and homophthalaldehyde yield, respectively, 2-nitroindenol, 2-nitrobenzindenol, and 2-nitronaphthalene. – Application of the method to sugar dialdehydes (aldehydic diglycol derivatives of monosaccharides formed by periodate oxidation) constitutes an excellent synthesis of 3-amino sugars, since 3-deoxy-3-nitropyranoses are formed smoothly on cyclization, and the corresponding 3-amino derivatives are obtained by hydrogenation. Thus, the reaction sequence: periodate oxidation → cyclization with nitromethane → hydrogenation, leads in the case of α- and β-D-pentosides to 3-amino-3-deoxy-D- and -L-pentosides, respectively, with ribo, xylo, and arabino configurations. α-D-Hexosides afford 3-amino-3-deoxy derivatives of glucose, mannose, and talose, while β-D-hexosides give derivatives with gluco, manno, and galacto configurations. 3-Amino-3,6-dideoxyglucosides of the D- and L-series are obtained from 6-deoxy-D- or -L-hexosides, respectively, and 3-aminohexosans with gulo, ido, and altro configurations are obtained from 1,6-anhydro sugars. Cyclization of the dialdehydes obtained from sedoheptulose and methyl 4,6-O-ethylidene-α-D-glucoside by periodate oxidation, leads to 3-nitro and, after hydrogenation, to 3-amino derivatives of 3-deoxyheptopyranoses.

Introduction

The base-catalysed condensation of an aldehyde with nitromethane, a reaction analogous to the aldol condensation, has been used for the synthesis of a variety of products since its discovery by *Henry* [2] in 1895. The primary products of the reaction are *aci*-nitro salts *(1)*. Neutralization of the latter with weak acids results in a

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[1] Extended version of lectures given at the Department of Biochemistry, University of California, Berkeley (May, 1961), the Department of Agricultural Chemistry, University of Kyoto (July, 1961), the meeting of chemistry lecturers at Bonn (September, 1962), and at the Technische Hochschule, Darmstadt (November, 1961 and December, 1962).

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